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(54) DNA conferring L-homoserine resistance to bacteria, and its use

(57) A bacterium which has an ability to produce an amino acid and in which a novel gene (*rhtB*) coding for a protein having an activity of making a bacterium having the protein L-homoserine-resistant is enhanced, is cultivated in a culture medium to produce and accumulate the amino acid in the medium, and the amino acid is recovered from the medium.

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DescriptionTechnical Field

5 [0001] The present invention relates to a method for producing an amino acid, especially for a method for producing L-homoserine, L-alanine, L-isoleucine, L-valine, or L-threonine using a bacterium belonging to the genus *Escherichia*.

Background Art

10 [0002] The present inventors obtained, with respect to *E. coli* K-12, a mutant having mutation, *thrR*, (herein referred to as *rhtA23*) that is concerned in high concentrations of threonine (>40 mg/ml) or homoserine (>5 mg/ml) in a minimal medium (Astaurova, O. B. et al., Appl. Bioch. and Microbiol., 21, 611-616 (1985)). On the basis of *rhtA23* mutation an improved threonine-producing strain (SU patent No. 974817), homoserine- and glutamic acid-producing strains (Astaurova et al., Appl. Bioch. And Microbiol., 27, 556-561 (1991)) were obtained.

15 [0003] Furthermore, the present inventors have revealed that the *rhtA* gene exists at 18 min on *E. coli* chromosome and that the *rhtA* gene is identical to ORF1 between *pexB* and *ompX* genes. The unit expressing a protein encoded by the ORF1 has been designated as *rhtA* (*rht*: resistance to homoserine and threonine) gene. The *rhtA* gene includes a 5'-noncoding region including SD sequence, ORF1 and a terminator. Also, the present inventors have found that a wild type *rhtA* gene participates in resistance to threonine and homoserine if cloned in a multicopy state and that enhancement of expression of the *rhtA* gene improves amino acid productivity of a bacterium belonging to the genus *Escherichia* having an ability to produce L-lysine, L-valine or L-threonine (ABSTRACTS of 17th International Congress of Biochemistry and Molecular Biology in conjunction with 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457).

20 [0004] It was found that at least two different genes which impart homoserine resistance in a multicopy state exist in *E. coli* during cloning of the *rhtA* gene. One of the genes is the *rhtA* gene, however the other gene has not yet been elucidated.

Disclosure of the Invention

30 [0005] An object of the present invention is to provide a novel gene participating in resistance to homoserine, and a method for producing an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine with a high yield.

[0006] The inventors have found that a region at 86 min on *E. coli* chromosome, when cloned by a multicopy vector, impart resistance to L-homoserine to cells of *E. coli*, and the amplification of this region results, like the *rhtA* gene, in an improvement of the amino acid productivity of *E. coli*. On the basis of these findings, the present invention has been completed.

[0007] Thus, the present invention provides:

- 40 (1) a protein comprising an amino acid sequence shown in SEQ ID No. 2 of the Sequence Listing or including deletion, substitution, insertion and/or addition of one or more amino acids in said sequence and having the activity of making a bacterium resistant to L-homoserine,
 (2) the DNA coding for a protein according to (1), which is a DNA defined in the following (a) or (b):

45 (a) a DNA which comprises a nucleotide sequence corresponding to the nucleotide numbers of 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing; or

(b) a DNA which hybridizes with the nucleotide sequence corresponding to the nucleotide numbers of 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing under stringent conditions, and which codes for the protein having the activity of making the bacterium resistant to L-homoserine,

50 (3) a bacterium belonging to the genus *Escherichia*, wherein L-homoserine resistance of the bacterium is enhanced by amplifying the copy number or increasing the expression rate of the DNA of (2) in a cell of the bacterium,

(4) the bacterium of (3), wherein the DNA of (1) is carried on a multicopy vector in the cell of the bacterium,

(5) the bacterium of (3), wherein the DNA of (2) is carried on a transposon in the cell of the bacterium,

55 (6) a method for producing an amino acid, comprising the steps of cultivating the bacterium of any of (3) to (5), which has an ability to produce the amino acid, in a culture medium to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium, and

(7) the method of (6), wherein the amino acid is at least one selected from the group consisting of L-homoserine,

L-alanine, L-isoleucine, L-valine and L-threonine.

[0008] The DNA of the present invention may be referred to as "*rhtB* gene", a protein coded by the *rhtB* gene may be referred to as "RhtB protein", an activity of the RhtB protein which participates in resistance to L-homoserine of a bacterium (i.e. an activity of making a bacterium having the RhtB protein L-homoserine-resistant) may be referred to as "Rh activity", and a structural gene encoding the RhtB protein in the *rhtB* gene may be referred to as "*rhtB* structural gene". The term "enhancing the Rh activity" means imparting resistance to homoserine to a bacterium or enhance the resistance by means of increasing the number of molecules of the RhtB protein, increasing a specific activity of the RhtB protein, or desensitizing negative regulation against the expression or the activity of the RhtB protein or the like.

10 The terms "DNA coding for a protein" mean a DNA of which one of strands codes for the protein when the DNA is double-stranded. The L-homoserine resistance means a property that a bacterium grows on a minimal medium containing L-homoserine at a concentration at which a wild type strain thereof can not grow, usually at 10 mg/ml. The ability to produce an amino acid means a property that a bacterium produces and accumulates the amino acid in a medium in a larger amount than a wild type strain thereof.

15 [0009] According to the present invention, resistance to homoserine of a high concentration can be imparted to a bacterium belonging to the genus *Escherichia*. The cultivation of a bacterium belonging to the genus *Escherichia*, which has increased resistance to homoserine and an ability to accumulate an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine or L-threonine in a medium results in a high yield of the desired amino acid.

[0010] The present invention will be explained in detail below.

20 (1) DNA of the present invention

[0011] The DNA of the present invention coding for a protein having the Rh activity and having an amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing. Specifically, the DNA of the present invention may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 557 to 1171 of a nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing.

[0012] The DNA of the present invention includes a DNA fragment encoding the RhtB protein conferring on the bacterium *Escherichia coli* resistance to homoserine, which includes the regulatory elements of the *rhtB* gene and the structural part of *rhtB* gene, having the nucleotide sequence shown in SEQ ID NO: 1.

30 [0013] The nucleotide sequence shown in SEQ ID NO: 1 corresponds to a part of sequence complement to the sequence of GenBank accession number M87049. SEQ ID NO: 1 includes f138 (nucleotide numbers 61959-61543 of GenBank accession number M87049) which is a known but function-unknown ORF (open reading frame) present at 86 min on *E. coli* chromosome, and 5'-flanking and 3'-flanking regions thereof. The f138, which has only 160 nucleotides in the 5'-flanking region, can not impart the resistance to homoserine. No termination codon is present between the 62160 and 61959 of M87049 (upstream the ORF f138). Hence, the coding region is 201 bp longer. Thus the RhtB protein and the *rhtB* gene coding for the protein are novel.

35 [0014] The *rhtB* gene may be obtained, for example, by infecting Mucis lysogenic strain of *E. coli* using a lysate of a lysogenic strain of *E. coli* such as K12 or W3110 according to the method in which mini-Mu d5005 phagemid is used (Groisman, E. A., et al., J. Bacteriol., 168, 357-364 (1986)), and isolating plasmid DNAs from colonies growing on a minimal medium containing kanamycin (40 µg/ml) and L-homoserine (10 mg/ml). As illustrated in the Example described below, the *rhtB* gene was mapped at 86 min on the chromosome of *E. coli*. Therefore, the DNA fragment including the *rhtB* gene may be obtained from the chromosome of *E. coli* by colony hybridization or PCR (polymerase chain reaction, refer to White, T.J. et al, Trends Genet. 5, 185(1989)) using oligonucleotide(s) which has a sequence corresponding to the region near the portion of 86 min on the chromosome of *E. coli*. Alternatively, the oligonucleotide

40 may be designed according to the nucleotide sequence shown in SEQ ID NO: 1. By using oligonucleotides having nucleotide sequences corresponding to an upstream region from the nucleotide number 557 and a downstream region from the nucleotide number 1171 in SEQ ID NO: 1 as the primers for PCR, the entire coding region can be amplified.

45 [0015] Synthesis of the oligonucleotides can be performed by an ordinary method such as a phosphoramidite method (see Tetrahedron Letters, 22, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.), using *Taq* DNA polymerase (supplied by Takara Shuzo Co., Ltd.) in accordance with a method designated by the supplier.

50 [0016] The DNA coding for the RhtB protein of the present invention may code for RhtB protein including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions, provided that the Rh activity of RhtB protein encoded thereby is not deteriorated. The DNA, which codes for the substantially same protein as the RhtB protein as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site

involve deletion, substitution, insertion or addition. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating a DNA coding for the RhtB protein *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus *Escherichia* harboring a DNA coding for the RhtB protein with ultraviolet irradiation or a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid usually used for the mutation treatment.

[0017] The DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by expressing a DNA subjected to *in vitro* mutation treatment as described above in multicopy in an appropriate cell, investigating the resistance to homoserine, and selecting the DNA which increases the resistance. Also, it is generally known that an amino acid sequence of a protein and a nucleotide sequence coding for it may be slightly different between species, strains, mutants or variants, and therefore the DNA, which codes for substantially the same protein, can be obtained from L-homoserine-resistant species, strains, mutants and variants belonging to the genus *Escherichia*. Specifically, the DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing under stringent conditions, and which codes for a protein having the Rh activity, from a bacterium belonging to the genus *Escherichia* which is subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus *Escherichia*. The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized.

(2) Bacterium belonging to the genus *Escherichia* of the present invention

[0018] The bacterium belonging to the genus *Escherichia* of the present invention is a bacterium belonging to the genus *Escherichia* of which the Rh activity is enhanced. A bacterium belonging to the genus *Escherichia* is exemplified by *Escherichia coli*. The Rh activity can be enhanced by, for example, amplification of the copy number of the *rhtB* structural gene in a cell, or transformation of a bacterium belonging to the genus *Escherichia* with a recombinant DNA in which a DNA fragment including the *rhtB* structural gene encoding the RhtB protein is ligated with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*. The Rh activity can be also enhanced by substitution of the promoter sequence of the *rhtB* gene on a chromosome with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*.

[0019] The amplification of the copy number of the *rhtB* structural gene in a cell can be performed by introduction of a multicopy vector which carries the *rhtB* structural gene into a cell of a bacterium belonging to the genus *Escherichia*. Specifically, the copy number can be increased by introduction of a plasmid, a phage or a transposon (Berg, D.E. and Berg, C.M., *Bio/Technol.*, 1, 417 (1983)) which carries the *rhtB* structural gene into a cell of a bacterium belonging to the genus *Escherichia*.

[0020] The multicopy vector is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, and phage vectors such as λ 1059, λ BF101, M13mp9 or the like. The transposon is exemplified by Mu, Tn10, Tn5 or the like.

[0021] The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. M. Morrison (*Methods in Enzymology* 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., *J. Mol. Biol.*, 53, 159 (1970)) and the like.

[0022] If the Rh activity is enhanced in an amino acid-producing bacterium belonging to the genus *Escherichia* as described above, a produced amount of the amino acid can be increased. As the bacterium belonging to the genus *Escherichia* in which the Rh activity is to be enhanced, strains which have abilities to produce desired amino acids are used. Besides, the ability to produce an amino acid may be imparted to a bacterium in which the Rh activity is enhanced. Examples of amino acid-producing bacteria belonging to the genus *Escherichia* are described below.

(1) L-threonine-producing bacteria

[0023] The L-threonine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain MG442 (Guaytiner et al., *Genetika* (in Russian), 14, 947-956 (1978)).

(2) L-homoserine-producing bacteria

[0024] The L-homoserine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain NZ10 (*thrB*). This strain was derived from the known strain C600 (*thrB*, *leuB*) (Appleyard R.K., *Genetics*, 39, 440-452).

(1954)) as Leu⁺ revertant.

[0025] On the basis of the *rhtB* DNA fragment, new amino acid-producing strains *E. coli* NZ10/pAL4,pRhtB; *E. coli* MG442/pVIC40,pRhtB; and *E. coli* MG442/pRhtB were obtained which are used for the production of amino acids by fermentation.

5 [0026] The new strains have been deposited (according to international deposition based on Budapest Treaty) in the Russian National Collection of Industrial Microorganisms (VKPM) on October 6, 1998. The strain *E. coli* NZ10/pAL4,pRhtB has been deposited as an accession number of VKPM B-7658; the strain *E. coli* MG442/pRhtB has been deposited as an accession number of VKPM B-7659; and the strain *E. coli* MG442/pVIC40,pRhtB has been deposited as an accession number of VKPM B-7660.

10 [0027] The strain *E. coli* NZ10/pAL4,pRhtB (VKPM B-7658) exhibits the following cultural-morphological and biochemical features.

Cytomorphology. Gram-negative weakly-motile rods having rounded ends. Longitudinal size, 1.5 to 2 μ m.

15 Cultural features:

[0028]

20 Beef-extract agar. After 24-hour growth at 37°C, produces round whitish semitransparent colonies 1.5 to 3 mm in diameter, featuring a smooth surface, regular or slightly wavy edges, the center is slightly raised, homogeneous structure, pastelike consistency, readily emulsifiable.

Luria's agar. After a 24-hour growth at 37°C, develops whitish semitranslucent colonies 1.5 to 2.5 mm in diameter having a smooth surface, homogeneous structure, pastelike consistency, readily emulsifiable.

25 Minimal agar-doped medium M9. After 40 to 48 hours of growth at 37°C, forms colonies 0.5 to 1.5 mm in diameter, which are colored greyish-white, semitransparent, slightly convex, with a lustrous surface.

Growth in a beef-extract broth. After 24-hour growth at 37°C, exhibits strong uniform cloudiness, has a characteristic odor.

Physiological and biochemical features:

30

[0029]

Grows upon thrust inoculation in a beef-extract agar.

Exhibits good growth throughout the inoculated area.

35 The microorganism proves to be a facultative anaerobe.

It does not liquefy gelatin.

Features a good growth on milk, accompanied by milk coagulation.

Does not produce indole.

40 Temperature conditions. Grows on beef-extract broth at 20-42°C, an optimum temperature lying within 33-37°C.

pH value of culture medium. Grows on liquid media having the pH value from 6 to 8, an optimum value being 7.2.

Carbon sources. Exhibits good growth on glucose, fructose, lactose, mannose, galactose, xylose, glycerol, and mannitol to produce an acid and gas.

Nitrogen sources. Assimilates nitrogen in the form of ammonium, nitric acid salts, as well as from some organic compounds.

45 Resistant to ampicillin, kanamycin and L-homoserine.

L-Threonine is used as a growth factor.

Content of plasmids. The cells contain multicopy hybrid plasmid pAL4 ensuring resistance to ampicillin and carrying the gene *thrA* of the threonine operon, which codes for aspartate kinase-homoserine dehydrogenase I responsible for the increased homoserine biosynthesis. Besides, the cells contain a multicopy hybrid plasmid pRhtB

50 ensuring resistance to kanamycin and carrying the *rhtB* gene which confers resistance to homoserine (10 mg/l).

[0030] The strain *E. coli* MG442/pRhtB (VKPM B-7659) has the same cultural-morphological and biochemical features as the strain NZ10/pAL4,pRhtB except for L-isoleucine is used as a growth factor instead of L-threonine. However, the strain can grow slowly without isoleucine. Besides, the cells of the strain contain only one multicopy hybrid plasmid pRhtB ensuring resistance to kanamycin and carrying the *rhtB* gene which confers resistance to homoserine (10 mg/l).

55 [0031] The strain *E. coli* MG442/pVIC40,pRhtB (VKPM B-7660) has the same cultural-morphological and biochemical features as the strain NZ10/pAL4,pRhtB except for L-isoleucine is used as a growth factor instead of L-threonine. However, the strain can grow slowly without isoleucine. The cells of the strain contain multicopy hybrid plasmid pVIC40

ensuring resistance to streptomycin and carrying the genes of the threonine operon. Besides, they contain multicopy hybrid plasmid pRhtB ensuring resistance to kanamycin and carrying the *rhtB* gene which confers resistance to homoserine (10 mg/l).

(3) Method for producing an amino acid

[0032] An amino acid can be efficiently produced by cultivating the bacterium in which the Rh activity is enhanced by amplifying a copy number of the *rhtB* gene as described above, and which has an ability to produce the amino acid, in a culture medium, producing and accumulating the amino acid in the medium, and recovering the amino acid from the medium. The amino acid is exemplified preferably by L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

[0033] In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino acid from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyte and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

[0034] The cultivation is preferably culture under an aerobic condition such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the accumulation of the target amino acid in the medium.

[0035] Recovering the amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying the target amino acid by ion exchange, concentration and crystalline fraction methods and the like.

Brief Explanation of Drawing

[0036]

Fig. 1 shows cloning, identification and inactivation of the *rhtB* gene.
Fig. 2 shows the amino acid sequence of the RhtB protein.

Examples

[0037] The present invention will be more concretely explained below with reference to Examples. In the Examples, an amino acid is of L-configuration unless otherwise noted.

Example 1: Obtaining of *rhtB* DNA fragment

(1) Cloning of *rhtB* gene into mini-Mu phagemid

[0038] The wild-type *rhtB* gene was cloned *in vivo* using mini-Mu d5005 phagemid (Groisman, E. A., et al., J. Bacteriol., 168, 357-364 (1986)). MuCts62 lysogen of the strain MG442 was used as a donor. Freshly prepared lysates were used to infect a Mucts lysogenic derivative of a strain VKPM B-513 (Hfr K10 metB). The cells were plated on M9 glucose minimal medium with methionine (50 µg/ml), kanamycin (40 µg/ml) and homoserine (10 mg/ml). Colonies which appeared after 48 hr were picked and isolated. Plasmid DNA was isolated and used to transform the strain VKPM B-513 by standard techniques. Transformants were selected on L-broth agar plates with kanamycin as above. Plasmid DNA was isolated from those which were resistant to homoserine, and analyzed by restriction mapping of the structure of the inserted fragments. It appeared that two types of inserts belonging to different chromosome regions had been cloned from the donor. Thus, at least two different genes that is in multicopy and imparts resistance to homoserine exist in *E. coli*. One of the two type of inserts is the *rhtA* gene which has already been reported (ABSTRACTS of 17th International Congress of Biochemistry and Molecular Biology in conjugation with 1997 Annual Meeting of the American

Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997). Among the other of the two types of inserts, a fragment of a minimum length which imparts the resistance to homoserine is of 0.8 kb (Fig. 1).

(2) Identification of *rhtB* gene

[0039] The insert fragment was sequenced by the dideoxy chain termination method of Sanger. Both DNA strands were sequenced in their entirety and all junctions were overlapped. The sequencing showed that the insert fragment included f138 (nucleotide numbers 61543 to 61959 of GenBank accession number M87049) which was a known but function-unknown ORF (open reading frame) present at 86 min of *E. coli* chromosome and 201 bp of the upstream region thereof (downstream region in the sequence of M87049). The f138 which had only 160 nucleotides in the 5'-flanking region could not impart the resistance to homoserine. No termination codon is present upstream the ORF f138 between 62160 and 61959 nucleotides of M87049. Furthermore, one ATG following a sequence predicted as a ribosome binding site is present in the sequence. The larger ORF (nucleotide numbers 62160 to 61546) is designated as *rhtB* gene. The RhtB protein deduced from the gene is highly hydrophobic and contains 5 possible transmembrane segments.

Example 2: Production of homoserine-producing strain

[0040] Strain NZ10 of *E. coli* was transformed by a plasmid pAL4 which was a pBR322 vector into which the *thrA* gene coding for aspartokinase-homoserine dehydrogenase I was inserted, to obtain the strains NZ10/pAL4. The strain NZ10 is a *leuB*⁺-reverted mutant (*thrB*) obtained from the *E. coli* strain C600 (*thrB*, *leuB*) (Appleyard, Genetics, 39, 440-452 (1954)).

[0041] The *rhtB* gene was inserted to a plasmid pUK21 which is the known plasmid pUC19 in which a kanamycin resistance gene substituted for an ampicillin resistance gene (Vieira, J. and Messing, J., Gene, 100, 189-194 (1991)), to obtain pRhtB.

[0042] The strain NZ10/pAL4 was transformed with pUK21 or pRhtB to obtain strains NZ10/pAL4,pUK21 and NZ10/pAL4,pRhtB.

[0043] The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin and 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium having the following composition and containing 50 mg/l kanamycin and 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of homoserine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

Fermentation medium composition (g/L)

[0044]

Glucose	80
(NH ₄) ₂ SO ₄	22
K ₂ HPO ₄	2
NaCl	0.8
MgSO ₄ · 7H ₂ O	0.8
FeSO ₄ · 7H ₂ O	0.02
MnSO ₄ · 5H ₂ O	0.02
Thiamine hydrochloride	0.0002
Yeast Extract	1.0
CaCO ₃	30
(CaCO ₃ was separately sterilized.)	

[0045] The results are shown in Table 1. As shown in Table 1, the strain NZ10/pAL4,pRhtB accumulated homoserine in a larger amount than the strains NZ10/pAL4 and NZ10/pAL4,pUK21 in which the *rhtB* gene was not enhanced.

Table 1

Strain	OD ₅₆₀	Accumulated amount of homoserine (g/L)
NZ10/pAL4	16.4	3.1
NZ10/pAL4,pUK21	14.3	3.3
NZ10/pAL4,pRhtB	15.6	6.4

Example 3: Production of alanine, valine and isoleucine with pRhtB-introduced strain

[0046] *E. coli* strain MG442 is a known strain (Gusyatiner, et al., 1978, Genetika (in Russian), 14:947-956).

[0047] The strain MG442 was transformed with the plasmids pUK21 and pRhtB to obtain strains MG442/pUK21 and MG442/pRhtB.

[0048] The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 3 and containing 50 mg/l kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 40 hours with a rotary shaker. After the cultivation, accumulated amounts of alanine, valine and isoleucine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

[0049] The results are shown in Table 2. As shown in Table 2, the strain MG442/pRhtB accumulated each of alanine, valine and isoleucine in a larger amount than the strain MG442/pUK21 in which the *rhtB* gene was not enhanced.

Table 2

Strain	OD ₅₆₀	Accumulated amount (g/L)		
		Alanine	Valine	Isoleucine
MG442/pUK21	13.4	0.2	0.2	0.3
MG442/pRhtB	13.7	0.7	0.5	0.5

Example 4: Production of threonine-producing strain

[0050] The strain MG442 (Example 3) was transformed by introducing a known plasmid pVIC40 (U.S. Patent No. 5,175,107 (1992)) by an ordinary transformation method. Transformants were selected on LB agar plates containing 0.1 mg/ml streptomycin. Thus a novel strain MG442/pVIC40 was obtained.

[0051] The strain MG442/pVIC40 was transformed with pUK21 or pRhtB to obtain strains MG442/pVIC40,pUK21 and MG442/pVIC40,pRhtB.

[0052] The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin and 100 mg/l streptomycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 3 and containing 50 mg/l kanamycin and 100 mg/l streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

[0053] The results are shown in Table 3. As shown in Table 3, the strain MG442/pVIC40,pRhtB accumulated threonine in a larger amount than the strains MG442/pVIC40 and MG442/pVIC40,pUK21 in which the *rhtB* gene was not enhanced.

Table 3

Strain	OD ₅₆₀	Accumulated amount of threonine (g/L)
MG442/pVIC40	17	13.6
MG442/pVIC40,pUK21	16.3	12.9

Table 3 (continued)

Strain	OD ₅₆₀	Accumulated amount of threonine (g/L)
MG442/pVIC40,pRhtB	15.2	16.3

Example 5: Effect of *rhtB* gene inactivation and amplification on bacterium *E. coli* resistance to some amino acids and amino acid analogues

[0054] To inactivate the chromosomal *rhtB* gene the plasmid pNPZ46 was constructed (Fig. 1) on the basis of pUK21 vector. It harbors a DNA fragment from 86 min of *E. coli* chromosome, with the *rhtB* gene and 5'-flanking and 3'-flanking regions thereof. Then the *Clal-Eco47III* fragment of the pNPZ46 plasmid *rhtB* gene was substituted for *AsuII-BsrBI* fragment containing *cat* (Cm^R) gene of pACYC184 plasmid (Chang and Cohen, J. Bacteriol., 134, 1141-1156, 1978) giving the pNPZ47 plasmid (Fig. 1). To introduce the obtained insertionally inactivated *rhtB* gene into the chromosome of the *E. coli* strain N99 (the streptomycin-resistant derivative of the known strain W3350 (Campbell, Virology, 14, 22-33, 1961)), the method of Parker and Marinus was used (Parker, B. and Marinus, M. G., Gene, 73, 531-535, 1988). The substitution of the wild type allele for the inactivated one was proved by phage P1 transduction and by Southern hybridization (Southern, E. M., J. Mol. Biol., 98, 503-517, 1975).

[0055] Then the susceptibility of the thus obtained *E. coli* strain N99 *rhtB::cat*, of the initial strain N99 (*rhtB*⁺) and of its derivative transformed with pRhtB plasmid, N99/pRhtB, to some amino acids and amino acid analogues was tested. Overnight cultures of the strains grown in M9 minimal medium at 37°C with a rotary shaker (10^9 cfu/ml) were diluted 1:100 and grown for 5 hours under the same conditions. Then the log phase cultures thus obtained were diluted and about 10^4 of alive cells were applied to well-dried test plates with M9 agar containing doubling increments of amino acids or analogues. The minimum inhibitory concentration (MIC) of these compounds were examined after 40-46 h cultivation. The results are shown in Table 4.

Table 4

Substrate	MIC ($\mu\text{g/ml}$)		
	N99(<i>rhtB</i> ⁺)	N99/pRhtB	N99 <i>rhtB::cat</i>
1. L-homoserine	250	30000	125
2. L-threonine	30000	50000	30000
3. L-serine	5000	10000	5000
4. L-valine	0.5	1	0.5
5. AHVA	50	2000	25
6. AEC	10	25	10
7. 4-aza-DL-leucine	40	100	40

[0056] It follows from the Table 4 that multiple copies of *rhtB* besides homoserine conferred upon cells increased resistance to threonine, serine, valine, α -amino- β -hydroxyvaleric-acid (AHVA), S-(2-aminoethyl)-L-cysteine (AEC), and 4-aza-DL-leucine. The inactivation of the *rhtB* gene, on the contrary, increased the cell sensitivity to homoserine and AHVA. These results in conjunction with the data on homology of the RhtB protein to LysE lysine efflux transporter of *Corynebacterium glutamicum* (Vrljic et al., Mol. Microbiol., 22, 815-826, 1996) indicate the analogues function for the *rhtB* gene product. The presumed efflux transporters, RhtB, has specificity to several substrates (amino acids), or may show non-specific effects as a result of amplification.

SEQUENCE LISTING

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<120> DNA coding for protein which confers on bacterium Escherichia coli resistance to L-homoserine, and method for producing L-amino acids

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 180 185 190
 Met Leu Val Gly Ala Leu Leu Ala Ser Ala Arg His Ala
 10 195 200 205

15 Claims

1. A protein comprising an amino acid sequence shown in SEQ ID No. 2 of the Sequence Listing or including deletion, substitution, insertion and/or addition of one or more amino acids in said sequence and having the activity of making a bacterium resistant to L-homoserine.
2. A DNA sequence coding for a protein according to claim 1.
3. The DNA sequence according to claim 2 having the sequence of nucleotide numbers 557 to 1171 of the sequence shown in SEQ ID No. 1 of the Sequence Listing.
4. The DNA sequence according to claim 2 which hybridizes with the sequence of nucleotide numbers 557 to 1171 of the sequence shown in SEQ ID No. 1 of the Sequence Listing under stringent conditions.
5. A vector containing the DNA sequence according to any of the claims 2 to 4.
6. A bacterium, wherein resistance to L-homoserine is enhanced by increasing the concentration of the protein according to claim 1 in the cell of said bacterium.
7. The bacterium according to claim 6, wherein the expression rate of the gene containing the DNA sequence according to any of the claims 2 to 4 is increased and/or the copy number of the DNA sequence according to any of the claims 2 to 4 is amplified.
8. The bacterium according to claim 7, wherein said DNA is carried on a transposon.
9. A bacterium containing the vector according to claim 5.
10. A method for producing an amino acid comprising the step of cultivating the bacterium according to any of the claims 6 to 9 having the ability to produce the amino acid, in a culture medium, and producing and accumulating the amino acid in the medium.
11. The method according to claim 10, wherein said amino acid is at least one selected from the group consisting of L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

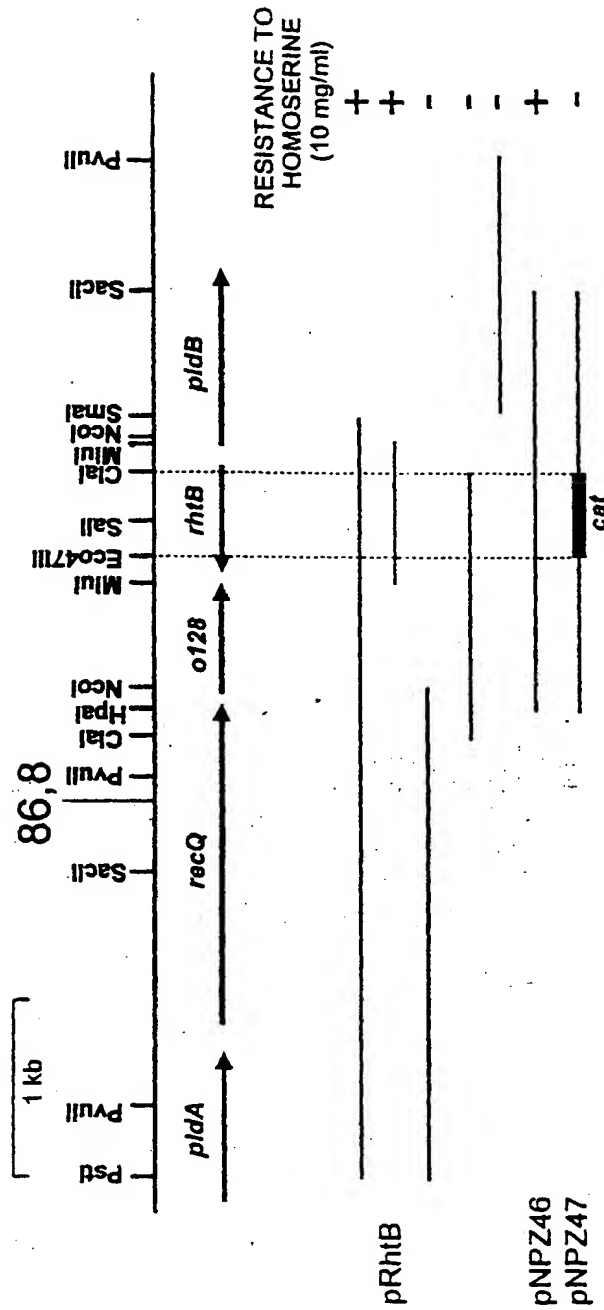


FIG. 1

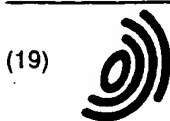
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(SEQ ID NO: 2)

FIG. 2



Europäisches Patentamt
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(11) **EP 0 994 190 A3**

(12)

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(54) **DNA conferring L-homoserine resistance to bacteria, and its use**

(57) A bacterium which has an ability to produce an amino acid and in which a novel gene (*rhtB*) coding for a protein having an activity of making a bacterium having the protein L-homoserine-resistant is enhanced, is

cultivated in a culture medium to produce and accumulate the amino acid in the medium, and the amino acid is recovered from the medium.

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European Patent
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EUROPEAN SEARCH REPORT

Application Number

EP 99 11 8581

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	DATABASE SMALL 'Online! 1 August 1992 (1992-08-01) DANIELS ET AL.: "Homoserine/homoserine lactone efflux protein" retrieved from EBI, accession no. RHTB_ECOLI Database accession no. P27847 XP002202582	1-5	C12N15/31 C07K14/245 C12N1/21 C12P13/06 C12P13/08 C12R1/19 C12P13/04
Y	* the whole document *	1-11	
X	-& DATABASE EM_PRO 'Online! EMBL; 29 January 1997 (1997-01-29) BLATTNER ET AL.: "Escherichia coli K12 MG1655 section 348 of 400 of the complete genome" retrieved from EBI, accession no. ECAE458 Database accession no. AE000458 XP002202583	1-5	
P,X	* the whole document * -& ZAKATAEVA ET AL.: "The novel transmembrane Escherichia coli proteins involved in the amino acid efflux" FEBS LETTERS, vol. 452, 11 June 1999 (1999-06-11), pages 228-232, XP002135075 * the whole document *	1-11	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C07K C12P
D,Y	ZAKATAEVA ET AL.: "Characterization of a pleiotropic mutation that confers upon Escherichia coli cells resistance to high concentrations of homoserine and threonine" FASEB JOURNAL, FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, US, vol. 11, no. 9, 31 July 1997 (1997-07-31), page a935 XP002135073 ISSN: 0892-6638 * the whole document *	1-11	
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 18 June 2002	Examiner Ceder, O
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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EUROPEAN SEARCH REPORT

Application Number
EP 99 11 8581

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	WO 97 23597 A (VRLIJC MARINA ;EGGELING LOTHAR (DE); SAHM HERMANN (DE); KERNFORSCH) 3 July 1997 (1997-07-03) * abstract; claims *	1-11	
A	US 5 017 483 A (FURUKAWA SATORU ET AL) 21 May 1991 (1991-05-21) * abstract *	1-11	
A	US 5 658 766 A (LIVSHITS VITALY ARKADIEVICH ET AL) 19 August 1997 (1997-08-19) * abstract *	1-11	
A	US 4 278 765 A (DEBABOV VLADIMIR G ET AL) 14 July 1981 (1981-07-14)		
P,X	ALESHIN V V ET AL: "A new family of amino-acid-efflux proteins" TIBS TRENDS IN BIOCHEMICAL SCIENCES, ELSEVIER PUBLICATION, CAMBRIDGE, EN, vol. 24, no. 4, 1 April 1999 (1999-04-01), pages 133-135, XP004214249 ISSN: 0968-0004 * the whole document *	1-11	
E	EP 1 013 765 A (AJINOMOTO KK) 28 June 2000 (2000-06-28) Seq Id Nos 1, 2 * abstract; claims *	1-11	
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 18 June 2002	Examiner Ceder, O
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 99 11 8581

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18-06-2002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9723597 A	03-07-1997	DE 19548222 A1	26-06-1997
		AU 724536 B2	21-09-2000
		AU 1921897 A	17-07-1997
		BR 9612666 A	05-10-1999
		CA 2241111 A1	03-07-1997
		CN 1209169 A	24-02-1999
		WO 9723597 A2	03-07-1997
		EP 0868527 A2	07-10-1998
		JP 2000507086 T	13-06-2000
		ZA 9610768 A	31-07-1998
US 5017483 A	21-05-1991	DE 3788583 D1	10-02-1994
		DE 3788583 T2	19-05-1994
		EP 0237819 A2	23-09-1987
		JP 2574786 B2	22-01-1997
		JP 63273487 A	10-11-1988
		KR 9108634 B1	19-10-1991
US 5658766 A	19-08-1997	US 5534421 A	09-07-1996
US 4278765 A	14-07-1981	SU 875663 A1	15-09-1982
		HU 190999 B	28-12-1986
EP 1013765 A	28-06-2000	RU 2148642 C1	10-05-2000
		AU 6543599 A	29-06-2000
		BR 9906283 A	03-04-2001
		CN 1260393 A	19-07-2000
		EP 1013765 A1	28-06-2000
		JP 2000189177 A	11-07-2000
		SK 184399 A3	11-07-2000
		ZA 9907819 A	30-06-2000

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82